(54) Title: MODIFIED HUMAN TNFALPHA (TUMOR NECROSIS FACTOR ALPHA) RECEPTOR

(57) Abstract

A polypeptide which is capable of binding human TNFα and which consists essentially of: a) the first three cysteine-rich subdomains, but not the fourth cysteine-rich subdomain, of the extracellular binding domain of the 55kD or 75kD receptor for human TNFα; or b) an amino acid sequence having a homology of 90 % or more with the said sequence (a).

- 1 -

Modified human TNFalpha(Tumor Necrosis Factor alpha) Receptor.

The present invention relates to recombinant proteins and their use.

Tumour necrosis factor-α (TNFα) is a potent cytokine
5 which elicits a broad spectrum of biological responses.
TNFα causes the cytolysis or cytostasis of many tumour cell
lines in vitro, induces the haemorrhagic necrosis of
transplanted tumours in mice, enhances the phagocytosis and
cytotoxicity of polymorphonuclear neutrophils, and
10 modulates the expression of many proteins, including
lipoprotein lipase, class I antigens of the major histo-
compatibility complex, and cytokines such as interleukin 1
and interleukin 6. TNFα appears to be necessary for a
normal immune response, but large quantities produce
15 dramatic pathogenic effects. TNFα has been termed
"cachectin" since it is the predominant factor responsible
for the wasting syndrome (cachexia) associated with
neoplastic disease and parasitemia. TNF is also a major
contributor to toxicity in gram-negative sepsis, since
20 antibodies against TNF can protect infected animals.

The many activities of TNFα are mediated by binding to a
cell surface receptor. Radioligand binding studies have
confirmed the presence of TNF receptors on a wide variety
of cell types. Although these receptors are expressed in
25 limited numbers (1,000 - 10,000 receptors/cell), they bind
TNFα with high affinity (Ka = $10^9 M^{-1}$ at 4°C). Lymphotoxin
(LT, also termed TNFβ) has similar, if not identical,
biological activities to TNFα, presumably because both are
recognized by the same receptor.

30 Recently, several laboratories have detected
heterogeneity in TNF receptor preparations. Two distinct
cell surface receptors which bind TNFα and TNFβ have
recently been characterised at the molecular level. cDNA
for one form of the receptor with a Mr of 55kD was isolated
35 utilising probes designed from the peptide sequence of a

- 2 -

soluble form of the receptor (1,2). A second receptor of
Mr 75kD was cloned by a COS cell expression approach (3).
Both receptors are members of a larger family of cytokine
receptors which include the nerve growth factor receptor,
5   the B cell antigen CD40, the rat T cell antigen MRC OX40.
In addition these receptors are homologous to the predicted
product of a transcriptionally active open reading frame
from shope fibroma virus which appears to give rise to a
secreted protein.

10   The most conserved feature amongst this group of cell
surface receptors is the cysteine rich extracellular ligand
binding domain, which can be divided into four repeating
motifs of about forty amino acids. We have now generated
four soluble receptor derivatives of the 55kD TNFα receptor
15   (TNFR). Each derivative is composed of the extracellular
binding domain but without one of the cysteine rich
subdomains. We have found that the derivative which lacks
the membrane-proximal fourth subdomain retains the ability
to bind TNFα with high affinity. This finding has general
20   applicability.

Accordingly, the present invention provides a
polypeptide which is capable of binding human TNFα and
which consists essentially of:

(a)   the first three cysteine-rich subdomains, but not
25   the fourth cysteine-rich subdomain, of the extracellular
binding domain of the 55kD or 75kD receptor for human TNFα;
or

(b)   an amino acid sequence having a homology of 90% or
more with the said sequence (a).

30   The invention also provides:
- a DNA sequence which encodes such a polypeptide;
- a vector which incorporates a DNA sequence of the
invention and which is capable, when provided in a
transformed host, of expressing the polypeptide of the
35   invention encoded by the DNA sequence; and

- 3 -

a host transformed with such a vector.

In the accompanying drawings:

Figure 1 shows the nucleotide sequence of the human TNFα cDNA and encoded amino acid sequence. The predicted signal

5   sequence residues are numbered -40 to -1. The transmembrane domain is boxed and potential N-linked glycosylation sites are overlined. The sequence homologous with the designed oligonucleotide probe is found at nucleotide positions 477-533.

10   Figure 2 is a Northern blot (lanes 1-3) of 10μg of oligo-dT selected RNA from human 293 cells (fibroblast cell line) (lane 1), placenta (lane 2) and spleen (lane 3) hybridised with the TNF receptor cDNA (Sma1-EcoRI fragment). The Southern blot (lanes 4-6) was hybridized

15   with the same probe. Human genomic DNA (5 μg per lane) was digested with Pst1 (lane 4), Hind III (lane 5) and EcoRI (lane 6).

Figure 3 shows the binding characteristics of recombinant human TNF receptor expressed in COS-7 cells.

20   The direct binding of recombinant ^{125}I-TNFα to COS-7 c..s transfected with prTNFR is presented in panel A. The inset contains Scatchard analysis derived from this data. As shown in panel B, monolayers of Cos-7 cells transfected with TNFR cDNA were incubated with 1nM ^{125}I-TNF in the

25   presence of various concentrations of unlabelled TNFα or TNFβ.

Figure 4 shows the effects of soluble TNFR on TNFα binding and biological activity. Panel A shows the effects of supernatants from Cos-7 cells transfected with a cDNA

30   encoding a soluble form of the TNF receptor (pTNFRecd, closed circles) or mock transfected (open circles) on ^{125}I-TNF binding to U937 cells. Panel B shows the effects of these supernatants on TNF mediated killing of WEHI 164 (clone 13) line. Assays were performed as described in

35   Materials and Methods.

- 4 -

Figure 5 is a diagram of the DNA sequence of pTNFRecd and is also a strategy map for polymerase chain reaction (PCR)-based domain deletion, in which 5'UTR is the 5'-untranslated region and I to IV are the four cysteine-rich
5 subdomains. The oligonucleotides employed in PCR in the Example and relevant restriction sites are also shown.

Figure 6 shows lined up the amino acid sequences of the four cysteine-rich subdomains of the 55kD (TNFR-55) and 75kD (TNFR-75) receptors and of rat nerve growth factor
10 receptor (NGFR), human CD40 and rat OX40. Homology is shown by means of boxes.

Figures 7 to 11 show the nucleotide sequence and the predicted amino acid sequence of the encoded polypeptide of pTNFRecd, pΔI, pΔII, pΔIII and pΔIV.
15 Figure 12 shows the results of the assays described in the Example 1.

Figure 13 shows diagrammatically the DNA encoding the 75kD receptor in which I to IV are the four cysteine-rich subdomains. Oligonucleotides employed in PCR-domain
20 deletion are also shown.

A polypeptide according to the invention is capable of binding human TNFα. Typically the polypeptide has a binding affinity for human TNFα of $10^7 M^{-1}$ or greater, for example $10^8 M^{-1}$ or greater. The affinity may be from 10^7 to
25 10^{10} M^{-1}, for example from 10^8 to $10^9 M^{-1}$.

A preferred polypeptide consists essentially of the first three cysteine-rich subdomains of the extracellular binding domain of the 55kD receptor for human TNFα. The sequence (a_1) of these three subdomains is: V   C   P   Q   G
30 K   Y   I   H   P   Q   N   N   S   I   C   C   T   K   C   H   K   G   T   Y
    L   Y   N   D   C   P   G   P   G   Q   D   T   D   C   R   E   C   E   S   G
    S   F   T   A   S   E   N   H   L   R   H   C   L   S   C   S   K   C   R   K
    E   M   G   Q   V   E   I   S· S   C   T   V   D   R   D   T   V   C   G   C
    R   K   N   Q   Y   R   H   Y   W   S   E   N   L   F   Q   C   F   N   C   S
35 L   C   L   N   G   T   V   H   L   S   C   Q   E   K   Q   N   T   V   C.

- 5 -

A useful polypeptide has the amino acid sequence (c):

```
    M  G  L  S  T  V  P  D  L  L  L  P  L  V  L  L  E  L  L  V
    G  I  Y  P  S  G  V  I  G  L  V  P  H  L  G  D  R  E  K  R
    D  S  V  C  P  Q  G  K  Y  I  H  P  Q  N  N  S  I  C  C  T
 5  K  C  H  K  G  T  Y  L  Y  N  D  C  P  G  P  G  Q  D  T  D
    C  R  E  C  E  S  G  S  F  T  A  S  E  N  H  L  R  H  C  L
    S  C  S  K  C  R  K  E  M  G  Q  V  E  I  S  S  C  T  V  D
    R  D  T  V  C  G  C  R  K  N  Q  Y  R  H  Y  W  S  E  N  L
    F  Q  C  F  N  C  S  L  C  L  N  G  T  V  H  L  S  C  Q  E
10  K  Q  N  T  V  C  T.
```

In an alternative embodiment, the polypeptide may consist essentially of the first three cysteine-rich subdomains of the extracellular binding domain of the 75kD receptor.

15   Apart from the amino acid sequence (a), the polypeptides may alternatively consist essentially of an amino acid sequence (b) having a homology of 90% or more with sequence (a). The degree of homology may be 95% or more or 98% or more. Amino acid sequence (a) may therefore be modified by

20 one or more amino acid substitutions, insertions and/or deletions and/or by an extension at either or each end. There should be no modification of the cysteine-residues, however. A polypeptide comprising sequence (b) must of course still be capable of binding human TNFα.

25   For example, one or more amino acid residues of the sequence (a), other than a cysteine residue, may be substituted or deleted or one or more additional amino acid residues may be inserted; provided the physicochemical character of the original sequence is preserved, i.e. in

30 terms of charge density, hydrophobicity/ hydrophilicity, size and configuration. Conservative substitutions may be made. Candidate substitutions are, based on the one-letter code (Eur. J. Biochem. 138, 9-37, 1984):

35 A for G and vice versa,

- 6 -

V by A, L or G;

K by R;

S by T and _vice versa_;

E for D and _vice versa_; and

5 Q by N and _vice versa_.

Up to 15 residues may be deleted from the N-terminal
and/or C-terminal of the polypeptide, for example up to 11
residues or up to 5 residues.

The polypeptides of the invention consist essentially of
10 sequence (a) or (b). They do not contain a fourth
cysteine-rich subdomain. However, the polypeptides may be
longer polypeptides of which sequence (a) or (b) is a part.
A short sequence of up to 50 amino acid residues may be
provided at either or each terminal of sequence (a) or (b).
15 The sequence may have up to 30, for example up to 20 or up
to 10, amino acid residues.

Alternatively, a much longer extension may be present at
either or each terminal of sequence (a) or (b) of up to,
for example, 100 or 200 amino acid residues. Longer amino
20 acid sequences may be fused to either or each end. A
chimaeric protein may be provided in which the or each
extension is a heterologous amino acid sequence, i.e. a
sequence not naturally linked to the amino acid sequence
above. Such a chimaeric protein may therefore combine the
25 ability to bind specifically to human TNFα with another
functionality.

The polypeptides of the invention lack the fourth
cysteine-rich subdomain of the 55kD or 75kD receptor as the
case may be. In particular, they lack the cysteine
30 residues of the fourth subdomain. They therefore do not
comprise, immediately after the third cysteine-rich
subdomain, any of the amino acid sequence up to the last
cysteine residue of the fourth cysteine-rich subdomain of
the relevant receptor except possibly the first amino acid
35 residue of that sequence. The polypeptides may extend

- 7 -

beyond that first amino acid residue as indicated above, though, by way of other amino acid sequences.

The polypeptides are typically recombinant polypeptides, although they may be made by synthetic methods such as
5   solid-phase or solution-phase polypeptide synthesis in which case an automated peptide synthesiser may be employed. They may therefore commence with a N-terminal residue M. They are prepared by recombinant DNA technology. The preparation of the polypeptides therefore
10 depends upon the provision of a DNA sequence encoding the polypeptide. A suitable sequence encoding the first three cysteine-rich subdomains of the extracellular binding domain of the 55kD receptor comprises: GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC AAG TGC
15 CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT
20 GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC.

A DNA sequence may further comprise a DNA sequence encoding a signal sequence fused to the 5' end of the coding sequence. Any signal sequence may be appropriate.
25 The signal sequence should be capable of directing secretion of the polypeptide of the invention from the cell in which the polypeptide is expressed. The signal sequence may be the natural signal sequence for the 55kD TNFα receptor. An appropriate DNA sequence encoding the first
30 three cysteine-rich subdomains of the extracellular binding domain of the 55kD receptor and such a signal sequence is therefore: ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT
35 GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT

- 8 -

TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT

CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC

TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC

TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC

5   ACA GTG GAC CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC

CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC

CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG

AAC ACC GTG TGC ACC.

A DNA sequence encoding a polypeptide of the invention

10   may be synthesised. Alternatively, it may be constructed

by isolating a DNA sequence encoding the 55kD or 75kD

receptor from a gene library and deleting DNA downstream of

the coding sequence for the first three cysteine-rich

subdomains of the extracellular binding domain of the

15   receptor. This gives DNA encoding the first three

subdomains of either receptor. As an intermediate step,

DNA encoding the entire or nearly the entire extracellular

binding domain may be isolated and digested to remove DNA

downstream of the coding sequence for the first three

20   subdomains.

A modified nucleotide sequence, for example encoding an

amino acid sequence (b), may be obtained by use of any

appropriate technique, including restriction with an

endonuclease, insertion of linkers, use of an exonuclease

25   and/or a polymerase and site-directed mutagenesis. Whether

a modified DNA sequence encodes a polypeptide of the

invention can be readily ascertained. The polypeptide

encoded by the sequence can be expressed in a suitable host

and tested for its ability to bind specifically human TNFα.

30   For expression of a polypeptide of the invention, an

expression vector is constructed. An expression vector is

prepared which comprises a DNA sequence encoding a

polypeptide of the invention and which is capable of

expressing the polypeptide when provided in a suitable

35   host. Appropriate transcriptional and translational

- 9 -

control elements are provided, including a promoter for the
DNA sequence, a transcriptional termination site, and
translational start and stop codons. The DNA sequence is
provided in the correct frame such as to enable expression
5 of the polypeptide to occur in a host compatible with the
vector.

The expression vector is then provided in an appropriate
host. Cells harbouring the vector are grown so as to
enable expression to occur. The vector may be a plasmid or
10 a viral vector. Any appropriate host-vector system may be
employed.

The transformed host may be a prokaryotic or eukaryotic
host. A bacterial or yeast host may be employed, for
example E. coli or S. cerevisiae. Insect cells can
15 alternatively be used, in which case a baculovirus
expression system may be appropriate. As a further
alternative, cells of a mammalian cell line, such as
Chinese Hamster Ovary (CHO) Cells may be transformed. A
polypeptide glycosylated at one, two or three of the sites
20 shown in Figure 1 can be obtained by suitable choice of the
host cell culture.

The polypeptide of the invention can be isolated and
purified. The N-terminal of the polypeptide may be
heterogeneous due to processing of the translation product
25 within a cell or as the product is being secreted from a
cell. A mixture of polypeptides according to the
invention, having different N-terminii, may therefore be
obtained. The polypeptide is soluble.

The polypeptides of the invention have activity binding
30 human TNFα. This activity is indictive of the possible use
of the polypeptides in the regulation of TNFα-mediated
responses by binding and sequestering human TNFα, for
example possible use in treatment of pulmonary diseases,
septic shock, HIV infection, malaria, viral meningitis,
35 graft versus host reactions and autoimmune diseases such as

- 10 -

rheumatoid arthritis.

For this purpose, a polypeptide of the present invention
may be formulated in a pharmaceutical composition. The
pharmaceutical composition also comprises a
5 pharmaceutically acceptable carrier or diluent.

The polypeptide of the invention may be administered to
a patient by any convenient route. The choice of whether
an oral route or a parenteral route, such as subcutaneous,
intravenous or intramuscular administration, is adopted; of
10 the dose; and of the frequency of administration depends
upon a variety of factors. These factors include the
purpose of the administration, the age and weight of the
patient being treated and the condition of the patient.
Typically, however, the polypeptide is administered in an
15 amount of from 1 to 1000 μg per dose, more preferably from
10 to 100 μg per dose, for each route of administration.

The following Examples illustrate the invention. A
Reference Example is provided.

REFERENCE EXAMPLE

20 1. Materials and Methods

   Reagents

Recombinant human TNFα and TNFβ were supplied as highly
purified proteins derived from E. coli. The specific
activities of these preparations were approximately 10^7
25 units/mg, as measured in the murine L929 cell cytotoxicity
assay (4). The synthetic oligonucleotides were prepared by
Oswel DNA Service (University of Edinburgh).

   Isolation of TNFα 55kD receptor cDNA clones

The sequence of a peptide fragment (E M G Q V E I S S T
30 V D R D T V C G) of the TNF binding protein was used to
design a synthetic oligonucleotide probe (5' AAG GAG ATG
GGC CAG GTT GAG ATC TCT TCT ACT GTT GAC AAT GAC ACT GTG TGT
GGC-3'). The 57-mer DNA probe was labelled with ^{32}P and T4

- 11 -

polynucleotide kinase (New England Biolab, Beverly, MA) and
used to screen a placenta cDNA library in gt10 (5,6).
Approximately 800,000 phage were transferred to
nitrocellulose filters and screened at reduced stringency
5   (7). Filters were incubated for 2 hours at 42°C in 0.05M
sodium phosphate, pH 6.5, 20% formamide, 0.75 M sodium
chloride, 0.075 M sodium citrate, 1% polyvinyl pyrrolidone
(Sigma, St Louis, MO), 1% Ficoll, 1% bovine serum albumin
(Sigma), and 50 ng/ml sonicated salmon sperm DNA (Sigma).
10 The radiolabelled probe was then added to the filters (10^8
cpm/ml final concentration) which were hybridized for 16
hours. Filters were washed extensively in 0.06M sodium
chloride, 0.006M sodium citrate, 1% SDS at 37°C and
positive clones were identified by autoradiography. Ten
15 hybridizing clones were plaque purified (5) and cDNA insert
size was determined by polyacrylamide gel electrophoresis
of EcoRI digested phage DNA. The inserts of two cDNA
clones were sequenced using the dideoxy chain termination
technique (8).

20   <u>Southern and Northern blot analysis</u>
     DNA was isolated from human lymphocytes by the method of
Blin and Stafford (9) and used for Southern blot analysis
(10). DNA was digested with restriction endonucleases (New
England Biolabs), fractionated on a 1% agarose gel, and
25 transferred to nitrocellulose. Hybridization and washing
were conducted under stringent conditions (6) using a ^{32}P-
labelled preparation of a 600 bp fragment of the TNF
receptor cDNA. Northern blot analysis was performed (11)
on oligo-dT selected RNA isolated from human placenta,
30 spleen (generously provided by the Cooperative Human Tissue
Network, Birmingham, AL) and a fibroblast cell line (293
cells). Following electrophoresis on a formaldehyde 1.2%
agarose gel, the RNA was transferred to nitrocellulose and
hybridized with the TNFα receptor DNA probe under stringent
35 conditions.

- 12 -

Mammalian cell expression of the human TNFα 55kD
receptor and derivatives

The coding region of the majority of the human TNFα 55kD
receptor was isolated as an EcoRI fragment and cloned into
5   a mammalian cell expression vector (12), resulting in
plasmid prTNFR. The EcoRI fragment encodes 374 amino acids
of the TNF receptor; the 81 carboxyl terminal residues of
the cytoplasmic domain are therefore missing from this
plasmid construction. A derivative of the TNFα receptor
10   was produced by engineering a termination codon just prior
to the transmembrane domain. The polymerase chain reaction
(PCR) technique (13) was used to generate a 300 bp
restriction fragment containing a BgIII site at the 5' end
and a HindIII site preceded by a TAG stop codon at the 3'
15   end. The PCR primers were 5'GCTGCTCCAAATGCCGAAAG and
5'AGTTCAAGCTTTTACAGTGCCCTTAACATTCTAA.

The PCR product was gel purified and cloned into the TNF
receptor expression plasmid (described above) digested with
BgIII and HindIII. DNA sequencing confirmed that the
20   resulting plasmid (pTNFRecd) contained the designed DNA
sequence. E. coli harbouring pTNFRecd were deposited at
the National Collection of Industrial and Marine Bacteria,
Aberdeen, GB on 11 September 1990 under accession number
NCIMB 40315.
25   The TNFα receptor expression plasmids were transfected
into monkey COS-7 cells using Lipofectin (Gibco BRL,
Bethesda, MD) according to the manufacturer's instructions.
Cells were cultured in Dulbecco's modified Eagle's medium
containing 10% fetal calf serum.

30   Analysis of recombinant TNFα 55kD receptor derivatives
TNFα was radioiodinated with the Iodogen method (Pierce)
according to the manufacturer's directions. The specific
activity of the ^{125}I-TNFα was 10-30 μCu/μg. COS cells

- 13 -

transfected with the TNFα receptor cDNA (prTNFR, 1300 bp
EcoRI fragment) were incubated for 24 hours and then seeded
into six well tissue culture plates (Nunc) at 4.5×10^8
cells per well. The cells were incubated for a further 48

5 hours and then receptor expression was quantitated by
radioligand binding for 2 hours at 4°C. Non-specific
binding of ^{125}I-TNFα was determined in the presence of a
1,000 fold molar excess of unlabelled TNFα. Binding data
was analysed by the method of Scatchard (14).

10   The TNFα receptor derivative was analysed for inhibition
of ^{125}I-TNFα binding to the natural receptor on human U937
cells. Culture supernatant was harvested 72 hours after
COS cells were transfected with pTNFRecd. U937 cells (2 x
10^8 cells in 200 µl) were incubated with 1nM ^{125}I-TNFα and

15 dilutions of COS cell media for 2 hours at 4°C. Cells were
then centrifuged through 20% sucrose to remove unbound
TNFα. Non-specific binding was determined in the presence
of 1µM unlabelled TNFα.

       The TNFα receptor derivative was also analyzed for

20 inhibition of TNFα cytotoxic effects <u>in vitro</u>. The
cytotoxicity assay was performed as described on the TNF
sensitive cell line WEHI 164 clone 13 (15). Serial
dilutions of supernatants from COS cells transfected with
pTNFRecd or mock transfected controls were incubated with a

25 constant amount of TNFα (1 ng/ml) for 1 hour at 27°C before
addition to the assay.

2. <u>RESULTS</u>
    <u>Isolation and characterization of the TNFα 55kD receptor</u>
<u>cDNA</u>

30   A partial amino acid sequence of the TNF binding protein
was used to design a synthetic oligonucleotide probe. The
radiolabelled probe was used to screen a human placenta
cDNA library in lambdagt10 and ten hybridizing phage were
isolated. The nucleotide and deduced amino acid sequences

- 14 -

of the longest cDNA clone are depicted in Figure 1. The
third potential ATG initiation codon occurs at position 156
of the nucleotide sequence; the first two ATG codons are
closely followed by termination codons, and the third ATG
5   is preceded by the best translation initiation consensus
nucleotides (16). The cDNA encodes an open reading frame
of 1365 bases which codes for a polypeptide of 455
residues. Both of the peptide sequences determined by
amino acid sequencing were identified in the encoded cDNA
10   (17 of 19 and 18 of 19 matching residues). The amino
terminal end identified for the TNF binding protein
corresponds to the cDNA encoded sequence beginning at
residue 41. The first 35 amino acids are generally quite
hydrophobic and probably represent a signal sequence.
15   Residues 35-40 are highly charged (DREKR) and such a
sequence is not typically found in secretory signal
sequences (17); perhaps the natural receptor is processed
by proteolysis after residue 40 which contains a dibasio
cleavage site (KR). Hydropathy analysis of the protein
20   sequence predicts a single transmembrane domain of 23 amino
acids. This hydrophobic sequence divides the protein into
an extracellular domain of 171 residues and a cytoplasmic
domain of 221 residues. The amino acid composition
determined for the TNF binding protein corresponds well
25   with the predicted composition of the extracellular domain
encoded by the cDNA (results not shown). The discrepancy
between the predicted receptor size (40,000 daltons) and
the size determined by SDS-polyacrylamide gel
electrophoresis (65,000 daltons, 18-20) is probably due to
30   glycosylation; there are four potential N-linked
glycosylation sites in the sequence, three of which are in
the extracellular domain. The sequence contains a large
number (17) of cysteine residues, 24 of which are in the
extracellular domain. The arrangement of these cysteine
35   residues is similar to that of several other cell surface

- 15 -

proteins, suggesting that the TNF receptor is structurally related to a family of receptors.

A Northern blot analysis is presented in Figure 2. The ^{32}P-labelled cDNA hybridized to a single predominant band
5 of oligo-dT selected RNA from human placenta or spleen. A minor larger transcript was also observed in RNA from a fibroblast cell line. The size of the hybridizing species is 2400 bases, in good agreement with the size of isolated cDNA. Also shown in Figure 2 is a Southern blot of human
10 genomic DNA hybridized with a 600 bp probe from the cDNA. In each of the three different restriction digests, only a single hybridized signal was observed. Thus the TNF receptor that we have isolated appears to be encoded by a single gene.

15

Expression of recombinant TNF receptor sequences in mammalian cells

To confirm that the cDNA shown in Figure 1 indeed encodes the TNF receptor, the cDNA was engineered for
20 expression in mammalian cells. The cDNA contains an EcoRI site at position 1270 of Figure 1. The receptor coding sequence was isolated as a 1300 bp EcoRI-fragment (containing all but the last 81 codons of the cytoplasmic domain) and inserted into a mammalian cell expression
25 vector containing a cytomegalovirus promoter and SV40 transcription termination sequences (12). The resulting plasmid was transfected into COS cells which were analyzed for TNF receptor expression after three days. As shown in Figure 3, the transfected cells specifically bound
30 radioiodinated TNFα in a saturable and dose dependent fashion. The population of COS cells expressed approximately 1×10^8 receptors per cell. The measured binding affinity of recombinant receptors was $2.5 \times 10^9 M^{-1}$ at 4°C which is in close agreement with natural receptor on
35 human cells (19,20). The binding of ^{125}I-TNFα(1 nM) to

- 16 -

these cells could be inhibited by the addition of
unlabelled TNFα or lymphotoxin (Figure 3b). COS cells
transfected with just the expression vector did not
significantly bind ^{125}I-TNFα (less than 2% of the binding
5 seen with the cDNA transfection).

The extracellular domain of the TNF receptor is
naturally shed from cells. To produce a similar
recombinant derivative, a stop codon preceding the
transmembrane domain was engineered into the cDNA by PCR
10 mutagenesis. The modified DNA was inserted into the
expression plasmid and subsequently transfected into COS
cells. After three days, the COS cell media was tested for
inhibition of TNFα binding to human U937 cells. As shown
in Figure 4a, the transfected cell media inhibited up to
15 70% of the binding of TNFα. The recombinant TNF receptor
derivative was next tested for inhibition of TNFα
biological activity. A sensitive bioassay for TNFα is a
measurement of cytolysis of mouse WEHI 164 (clone 13)
cells. The transfected cell media inhibited 60% of TNFα
20 cytotoxicity on this cell line (Figure 4b). Media from
mock transfected COS cells did not inhibit TNFα induced
cytotoxicity or binding. These experiments demonstrate
that the recombinant extracellular domain of the TNF
receptor is capable of binding TNF and inhibiting its
25 biological activity.

EXAMPLE 1: Expression of polypeptide consisting essentially
of the first three cysteine-rich subdomains of the
extracellular binding domain of the 55kD receptor
1. MATERIALS AND METHODS
30   Reagents
E. coli derived recombinant human TNFα had a specific
activity of 2×10^7 U/mg in an L929 cytotoxicity assay.
Oligonucleotides were purchased from Oswel DNA service
(University of Edinburgh).

- 17 -

Generation of the recombinant soluble TNFR derivatives

Deletion of each of the subdomains in the recombinant
soluble TNFR was achieved by means of PCR fragment joining
and PCR mutagenesis. The sequence of the oligonucleotides
5   used in these experiments is given in Table 1 and their
locations relative to the four cysteine rich subdomains is
shown in Figure 5. The four subdomains are lined up with
respect to one another in Figure 6.

The plasmid pTNFRecd (Reference Example) is shown in
10   Figure 7. pTNFRecd was further modified to remove 5'
untranslated sequences by cloning of the Cla I/Bgl II
digested product of a PCR using oligos 5' Cla and IIIA into
ClaI/Bgl II digested pTNFRecd, to generate 5'-ΔCla.
Digestion of 5'-ΔCla with Pst-1 and religation resulted in
15   the generation of pΔII, which lacks the second cysteine
rich subdomain (Figure 9). The fourth cysteine rich
subdomain was removed by cloning of the BglII/Hind ̄I̅I
digested product of a PCR using oligonucleotides 5A and 4D
into BglII/Hind III 5'-ΔCla; this introduced a termination
20   codon after amino acid 167 (counting from the initial
methionine) to yield pΔIV (Figure 11). The constructs p I
(Figure 8) and pΔIII (Figure 10) which lack the first and
third cysteine rich subdomains respectively were generated
by joining PCR fragments by means of overlaps introduced
25   into the primers used for the PCR. The gel purified
products of PCR's using 5' Cla and IA and IB and 5D were
mixed and subjected to further amplification using 5'Cla
and 5D as primers. The resulting fragment was digested
with ClaI and BglII and cloned into ClaI/BglII digested
30   pTNFRecd, to yield pΔI.

Similarly the ge ̄ purified products of PCR's using 5'
Cla and IIIA and IIIꞴ and 5D were mixed and subjected to
further amplification using 5'Cla and 5D as primers. This
product was digested with BglII and HindIII and cloned into
35   Bgl II/Hind III cut 5'-ΔCla to yield pΔIII. In all cases

- 18 -

the cloned derivatives were analysed by restriction enzyme analysis and DNA sequencing using sequenase (United States Biochemical Corporation).

Table 1: Structure of the mutagenic oligonucleotides

5 Oligo   Sequence
Name

5'Cla   5'-GTTCTATCGATAAGAGGCCATAGCTGTCTGGC-3'

IA   5'-GCTCTCACACTCTCTCTTCTCCCTGTCCCCTAG-3'

IB   5'-AGGGAGAAGAGAGAGTGTGAGAGCGGCTCCTTC-3'

10 IIIA   5'-TGCATGGCAGGTACACACGGTGTCCCGGTCCAC-3'

IIIB   5'-GACACCGTGTGTACCTGCCATGCAGGTTTCTTT-3'

4D   5'-GGCCAAGCTTCAGGTGCACACGGTGTTCTG-3'

5A   5'-GCTGCTCCAAATGCCGAAAG-3'

5D   5'-AGTTCAAGCTTTACAGTGCCCTTAACATTCTAA-3'

15 **Analysis of recombinant soluble TNFR derivatives**

COS cells were maintained in Dulbecco's modified Eagles medium containing 5% foetal calf serum. The soluble TNFα receptor derivatives were transfected into monkey COS cells by means of lipofectin (GIBCO-BRL, Bethesda MD) according

20 to the manufacturers protocol and cell free supernatants harvested 72 hours post transfection.

Inhibition of TNFα activity

The soluble TNFα receptor derivatives were analyzed for inhibition of TNFα cytotoxic activity *in vitro*. The

25 cytotoxicity assay was performed as described on the TNFα sensitive cell line WEHI 164 clone 13. Serial dilutions of supernatants from COS cells transfected with the mutant receptors or mock transfected controls were incubated with a constant amount of TNF (1 ng/ml) for 1 hour at 37°C

30 before addition to the assay.

2. **RESULTS**

In order to understand more about the contribution of

- 19 -

the individual cysteine rich subdomains to the binding of
TNFα by the soluble form of the 55kD TNF receptor, we
removed each subdomain by PCR mutagenesis (Figure 5). COS
cells were transfected with each of these constructs and

5   the supernatants were assayed for their ability to inhibit
the cytotoxic activity of TNFα. Figure 12 panel A shows
that conditioned medium from COS cells tranfected with
pTNFRecd inhibits TNFα as previously described. Removal of
the fourth cysteine rich subdomain resulted in a protein

10 which, similar to TNFRecd, was a potent inhibitor of TNFα
(Figure 12 panel B). The mutants lacking the first, second
and third subdomains did not show any inhibitory activity
in the TNFα cytotoxicity assay.

EXAMPLE 2: Expression of polypeptide consisting essentially

15 of the first three cysteine-rich subdomains of the
extracellular binding domain of the 75kD receptor.
The coding region of the human 75kD TNFα receptor was
isolated from a T cell lambda ZAP library, using a probe
based on published sequences (3) and cloned into the EcoRI

20 site of a mammalian cell expression vector (12) resulting
in plasmid p75TNFR. In more detail, RNA was extracted from
a cell line expressing the 75kD receptor and reverse
transcribed. Any cell line expressing this receptor could
be used, such as those described by Smith et al (3). The

25 product of the reverse transcription was subjected to 25
cycles of PCR using the following primers:
5' CGC AGA ATT CCC CGC AGC CAT GGC GCC CGT CGC C 3'
and 5' GTA AGG ATC CTA TCG CCA GTG CTC CCT TCA GCT 3'.
These primers are directed against the extracellular

30 binding domain coding region of the 75kD receptor and were
taken from Smith et al (3). The amplified product was gel
purified and shown to encode TNFR. This was subsequently
used to screen the library. Plaque purification was
performed essentially as described in the Reference Example

- 20 -

except that the probe was labelled by random priming (21)
and hybridised in 50% formamide. Filters were washed in
0.2 x SSC (Standard Saline Citrate) twice at 60°C.

A derivative of the 75kD TNFα receptor was produced by
5   engineering a termination codon just prior to the
transmembrane domain. Referring to Figure 13, the
polymerase chain reaction (PCR) technique was used to
generate a 274 bp restriction fragment containing a BglII
site at the 5' end and an Xba I site preceded by a TAG stop
10 codon at the 3' end. The PCR primers were 5'
ACACGACTTCATCCACGGATA and
5'ACGTTCTAGACTAGTCGCCAGTGCTCCCTTCAGCTG. The PCR product
was digested with Bgl II and Xba I, gel purified and cloned
into the TNF receptor expression plasmid (described above)
15 digested with BglII and Xba I. DNA sequencing confirmed
that the resulting plasmid contained the designed DNA
sequence.

A similar approach was utilised to generate a construct
which lacked the fourth cysteine-rich subdomain of the 75kD
20 TNFα receptor. PCR was performed using a primer upstream
of the Esp I site in the 75kD TNFR and a primer which
introduced a TAG termination codon and an Xba I site. The
sequences of the primers was 5' CAG AAC CGC ATC TGC ACC TGC
and 5'ACGTTCTAGACTTGCACACCACGTCTGATGTTTC respectively. The
25 PCR product was digested with EspI and Xba I and the 110bp
DNA fragment gel purified and cloned into Esp I Xba I
digested p75TNFR.

- 21 -

REFERENCES

1.   Loetscher, H., Pan, Y.-C.E., Lahm, H.-W., Gentz, R., Brockhaus, M., Tabuchi, H. and Lesslayer, W. (1990) Cell, 61, 351-359.

2.   Schall, T.J., Lewis, M., Koller, K.J., Lee, A., Rice, G.C., Wong, G.H.W., Gatanaga, T., Granger, G.A., Lentz, R., Raab, H., Kohl, W.J. and Goeddel, D.Y. (1990) Cell, 61, 361-370.

3.   Smith, C.A., Davis, T., Anderson, D., Solam, L., Beckmann, M.P., Jerzy, R., Dower, S.K., Cosman, D. and Goodwin, R.G. (1990) Science 248, 1019-1023.

4.   Ruff, M.R. & Gifford, G.E. (1981) Infection and Immunity, 31, 380.

5.   Maniatis, T., Hardison, R.C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G.K. and Efstratiadis, A. (1978) Cell 15, 687-701.

6.   Lawn, R.M., Fritsch, E.F., Parker, R.C., Blake, G & Maniatis, T. (1978) Cell 15, 1157-1174.

7.   Gray, P.W., Leong, S.R., Fennie, E., Farrar, M.A., Pingel, J.T. and Schreiber, R.D. (1989) Proc. Natl. Acad. Sci USA 86, 8497-8501.

8.   Smith, A.J.H., (1980) Meth. Enzym. 65 560-580.

9.   Blin, N, & Stanford, D.W. (1976) Nucl. Acids Res. 3, 2303-2398.

10.   Southern, E.M. (1975) J. Molec. Biol. 98, 503-517.

11.   Dobner, P.R., Kawasaki, E.S., Yu, L.Y. and Bancroft, F.C. (1981) Proc. Natl. Acad. Sci. USA. 78, 2230-2234.

12.   Eaton, D.L., Wood, W.I., Eaton, D., Hass, P.E., Hollinghead, P., Wion, K., Mather, J., Lawn, R.M., Vahar, G.A. and Gorman, C. (1986) Biochemistry 25: 8343-8347.

13.   Scharf, S.J., Horn, G.T., Erlich, H.A. (1986) Science 233, 1076-1079.

14.   Scatchard, G. (1949) Ann. New York Acad. Sci. 51, 660-672.

- 22 -

15. Espevik, T. & Nissen-Meyer, J. (1986) J. Immunol.
    Meths. 95, 99-105.

16. Kozak, M. (1989) J. Cell. Biol. 108, 229-241.

17. von Heijne, G. (1988) Nucl. Acids. Res. 14, 4683-4690.

18. Creasy, A.A., Yamamoto, R. & Vitt, C.R. (1987) Proc.
    Natl. Acad. Sci. USA. 84, 3293-3297.

19. Stauber, G.B., Alyer,R.A. & Aggarwal, B.B. (1988) J.
    Biol. Chem. 263, 19098-19104.

20. Scheurich, P., Ucer, U., Kronke, M. and Pfitzenmaier,
    K. (1986) Int. J. Cancer, 38, 127-133.

21. Feinburg, A. & Vogelstein, B (1984) Analytical
    Biochem. 137, 266-277.

- 23 -

CLAIMS

1.   A polypeptide which is capable of binding human TNFα and which consists essentially of:

(a)   the first three cysteine-rich subdomains, but not the
5 fourth cysteine-rich subdomain, of the extracellular binding domain of the 55kD or 75kD receptor for human TNFα; or

(b)   an amino acid sequence having a homology of 90% or more with the said sequence (a).

10   2.   A polypeptide according to claim 1, which consists essentially of the first three cysteiine-rich subdomains of the extracellular binding domain of the 55kD receptor for human TNFα.

3.   A polypeptide according to claim 2, which has the
15 amino acid sequence: M G L S T V P D L L L P L

V L L E L V G I Y P S G V I G L V P H L

G D R E K R D S V C P Q G K Y I H P Q N

N S I C C T K C H K G T Y L Y N D C P G

P G Q D T D C R E C E S G S F T A S E N

20 H L R H C L S C S K C R K E M G Q V E I

S S C T V D R D T V C G C R K N Q Y R H

Y W S E N L F Q C F N C S L C L N G T V

H L S C Q E K Q N T V C T.

4.   A DNA sequence which encodes a polypeptide as
25 defined in any one of the preceding claims.

5.   A DNA sequence according to claim 4, which comprises:

GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT

TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT

30 CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC

TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC

TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC

ACA GTG GAC CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC

CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC

- 24 -

CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG
AAC ACC GTG TGC.

      6.   A DNA sequence according to claim 4 or 5, which
further comprises a 5' sequence which encodes a signal
5 amino acid sequence.

      7.   A DNA sequence according to claim 4, which is:
ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC
CTG GAG CTG TTG GTG GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG
GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC
10 CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC
AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG
GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC
GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA TGC
CGA AAG GAA ATG GGT CAG GTG GAG ATC TT TCT TGC ACA GTG GAC
15 CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT
TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC
AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG
TGC ACC.

      8.   A vector which incorporates a DNA sequence as
20 claimed in any one of claims 4 to 7 and which is capable,
when provided in a suitable host, of expressing the said
polypeptide.

      9.   A vector according to claim 8, which is a
plasmid.

25   10.   A host transformed with a vector as claimed in
claim 8 or 9.

     11.   A host according to claim 10, which is a
mammalian cell line.

     12.   A process for the preparation of a polypeptide as
30 defined in claim 1, which process comprises culturing a
transformed host as claimed in claim 10 or 11 under such
conditions that the said polypeptide is expressed.

     13.   A pharmaceutical composition comprising a
pharmaceutically acceptable carrier or diluent and, as an

- 25 -

active principle, a polypeptide as claimed in claim 1.

14. A polypeptide as defined in claim 1 for use in the treatment of rheumatoid arthritis.
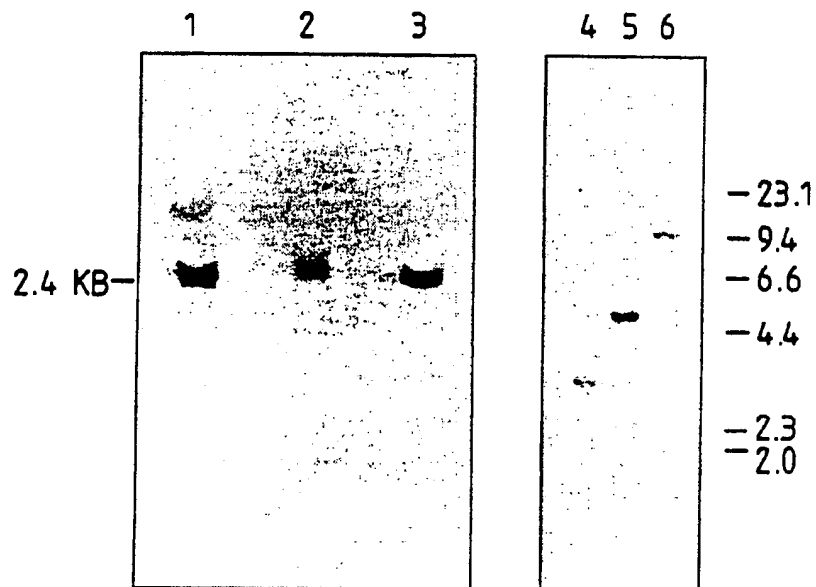
Fig. 1.

```
  1  ACCA GTGATCTCTA TGCCCGAGTC TCAACCCTCA ACTGTCACCC CAAGGCACTT GGACGTCCT GGACAGACCG
 75  AGTCCCCGGGA AGCCCCAGCA CTGCCGCTGC CACACTGCCC TGAGCCCAAA TGGGGGAGTG AGAGGCCATA GCTGTCTGGC

          M   G   L   S   T   V   P   D   L   L   L   P   L   V   G   I   Y   P
156       ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG GGA ATA TAC CCC

          S   G   V   I   G   L   V   P   H   L   G   D   R   E   K   G   K
228       TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG GGA AAG

          Y   I   H   P   Q   N   N   S   I   C   C   T   K   C
300       TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC AAG TGC

          P   G   P   G   Q   D   T   D   C   R   E   C   E   S   G   S   F   T   A   S   E   N   H   L
372       CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC

          R   H   C   L   S   C   S   K   C   R   K   N   Q   Y   R   H   V   E   I   S   C   V   D
444       AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG AAC CAG TAC CGG CAT GTG GAG ATC TCT TGC ACA GTG GAC

          R   D   T   V   C   C   G   R   K   N   Q   Y   R   H   Y   W   S   E   N   L   F   Q   C   F
516       CGG GAC ACC GTG TGT TGC GGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC

          N   C   S   L   C   L   N   G   T   V   H   L   S   C   Q   E   K   Q   N   C   K   S   L   E   C   T
558       AAT TGC AGC CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC TGT AAG AGC CTG GAG TGC ACG

          H   A   G   F   F   F   L   R   E   N   E   C   V   S   C   S   N   C   K   K   S
660       CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC TCC TGT AGT AAC TGT AAG AAG AGC

          K   L   C   L   P   Q   I   E   N   V   K   G   T   E   D   S   G   T   T  [V   L   L   P   L]
732       AAG TTG TGC CTA CCC CAG ATT GAG AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC ACA  GTG CTG TTG CCC CTG

         [V   I   F   F   G   L   C   L   L   S   L   F   I   G   L   M   Y]  R   Y   Q   R   W   K
804       GTC ATT TTC TTT GGT CTT TGC CTT TTA TCC CTC TTC ATT GGT TTA ATG TAT  CGC TAC CAA CGG TGG AAG

          S   K   L   Y   S   I   V   C   G   K   S   T   P   E   K   E   G   E   L   E   G   T   T   T
876       TCC AAG CTC TAC TCC ATT GTT TGT GGG AAA TCG ACA CCT GAA AAA GAG GGG GAG CTT GAA GGA ACT ACT ACT
```

Fig.1(cont.)

```
225 K   P   L   A   P   N   P   S   F   S   P   T   P   G   F   T   P   T   L   G   F   S   P   V
948 AAG CCC CTG GCC CCA AAC CCA AGC TTC AGT CCC ACT CCA GGC TTC ACC CCC ACC CTG GGC TTC AGT CCC GTG

249  P   S   S   T   F   T   S   S   T   Y   T   P   G   D   C   P   N   F   A   P   R   R
1020 CCC AGT TCC ACC TTC ACC TCC AGC TAT ACC GAC TGT CCC AAC TTT GCG CGC CGC AGA

273  E   V   A   P   P   Y   Q   G   A   D   P   L   A   T   L   A   S   D   P   I   P   N
1092 GAG GTG GCA CCA CCC TAT CAG GGG GCT GAC CCC CTC ACA GCC CTT GCG TCC GAC CCC ATC CCC AAC

297  P   L   Q   K   W   E   D   S   A   H   K   P   Q   S   L   D   T   D   D   P   A   T   L   Y
1164 CCC CTT CAG AAG TGG GAG GAC AGT GCC CAC AAG CCA CAG AGC CTA GAC ACT GAT GAC CCG GCG ACG CTG TAC

321  A   V   V   E   N   V   P   L   R   T   L   E   F   V   R   R   L   G   L   S   D   H   E
1236 GCC GTG GTG GAG AAC GTG CCC CCG TTG CGC TGG AAG GAA TTC GTG CGG CGC CTA GGG CTG AGC GAC CAC GAG

345  I   D   R   L   E   L   Q   N   G   R   C   L   R   E   A   Q   Y   S   M   L   A   T   W   R
1308 ATC GAT CGG CTG GAG CTG CAG AAC GGG CGC TGC CTG CGC GAG GCG CAA TAC AGC ATG CTG GCG ACC TGG AGG

369  R   R   T   P   R   R   E   A   T   L   E   L   L   G   R   V   L   R   N   M   D   L   L   G
1380 CGG CGC ACG CCG CGG CGG GAG GCC GAG CTG CTG GGA CGC GTG CTC CGC GAC ATG GAC CTG CTG GGC

393  C   L   E   D   I   E   E   A   L   C   G   P   A   A   L   P   P   A   P   S   L   L   R
1452 TGC CTG GAG GAC ATC GAG GAG GCG CTT TGC GGC CCC GCC GCG CTC CCG CCC GCG CCC AGT CTT CTC AGA TGA
1521 GGCTGCGCCC TGCGGGGCAGC TCTAAGGACC GTCCCTCGCAG ATCGCCTTCC AACCCCACTT TTTTCTGGAA AGGAGGGGTC
1601 CTGCAGGGGC AAGCAGGAGC TAGCAGCCGC CTAACCCCTC GATGTACATA GCTTTTCTCA GCTGCCTGCG
1681 CGCCGCCGAC AGTCAGCGCT GTGCGCGCGG AGAGAGGTGC GCCGTGGGCT CAAGAGCCTG AGTGGGTGGT TTGCGGAGGAT
1761 GAGGGACGCT ATGCCTCATG GTGTCCCTCAC CAGCAAGGCT GCTCGGGGGC CCCTGGTTCG TCCCTGAGCC
1841 TTTTTCACAG TGCATAAGCA GTTTTTTTTG TTTTTGTTTT GTTTTTTAAA TCAATCATGT TACACTAATA
1921 GAAACTTGGC ACTCCTGTGC CCTCTGCCTG GACAAGCAC ATAGCAAGCT GAACTGTCCT AGGCAGGGG CGAGCACGGA
2001 ACAATGGGGC CTTCAGCTGG AGCTGTGGAC TTTTGTACAT ACACTAAAAT TCTGAAGTTA AG
```
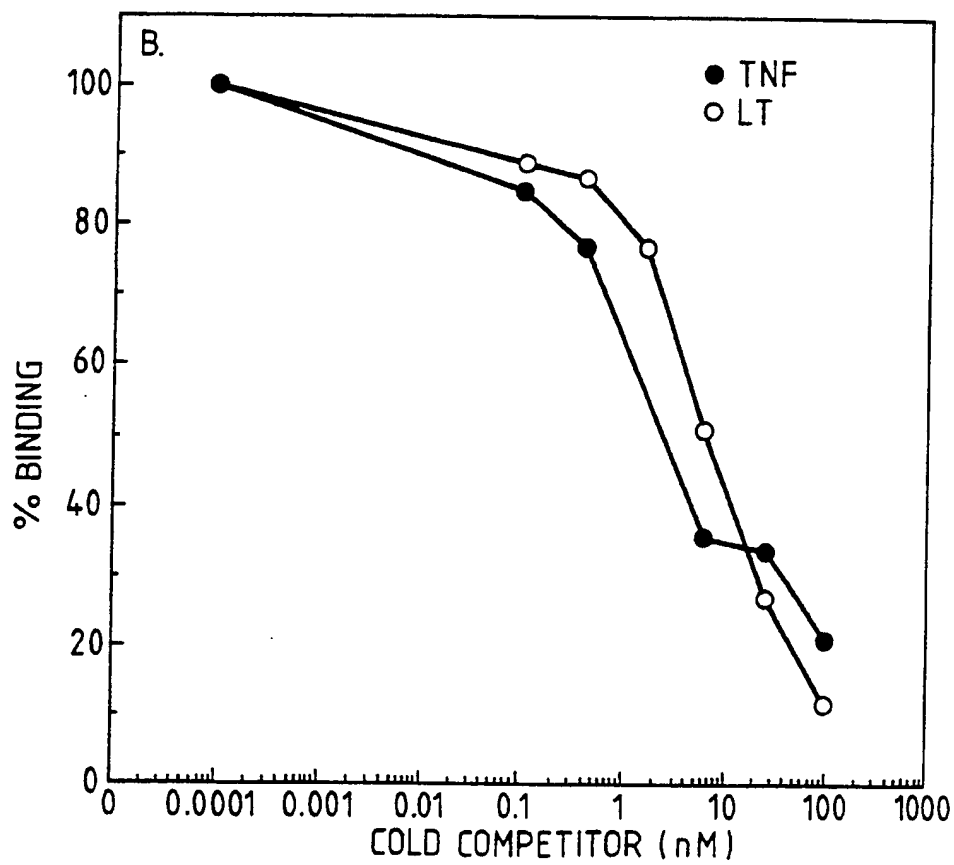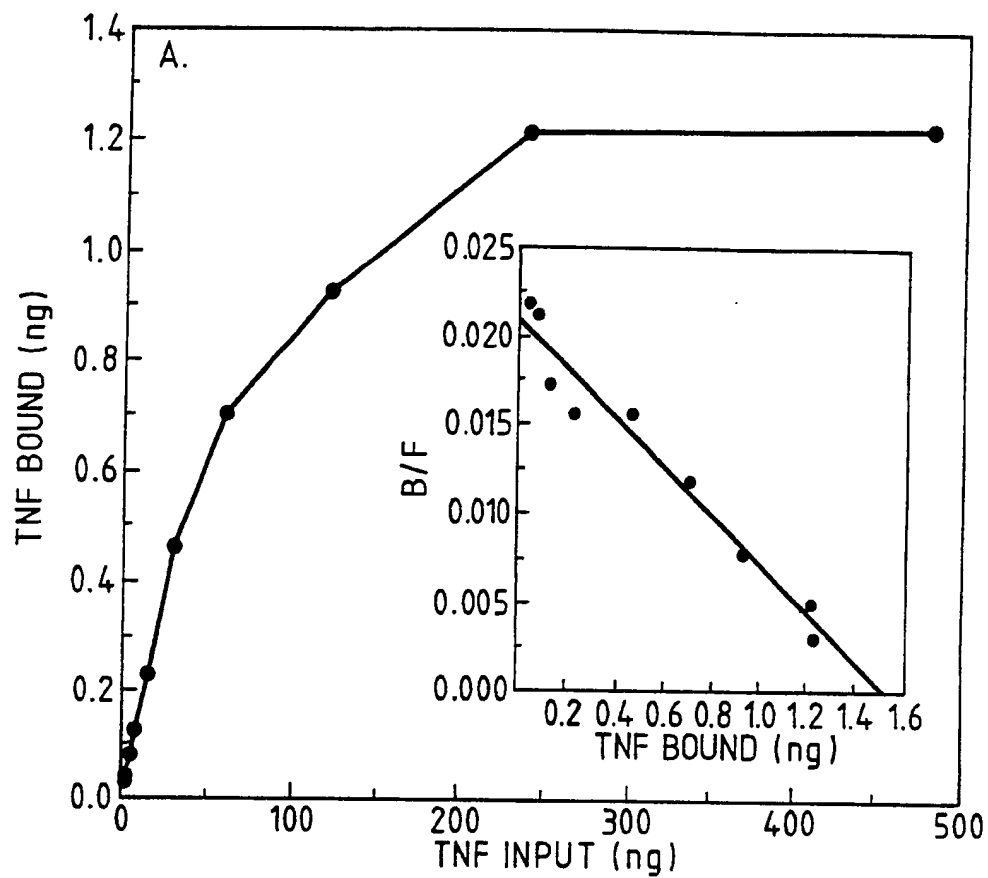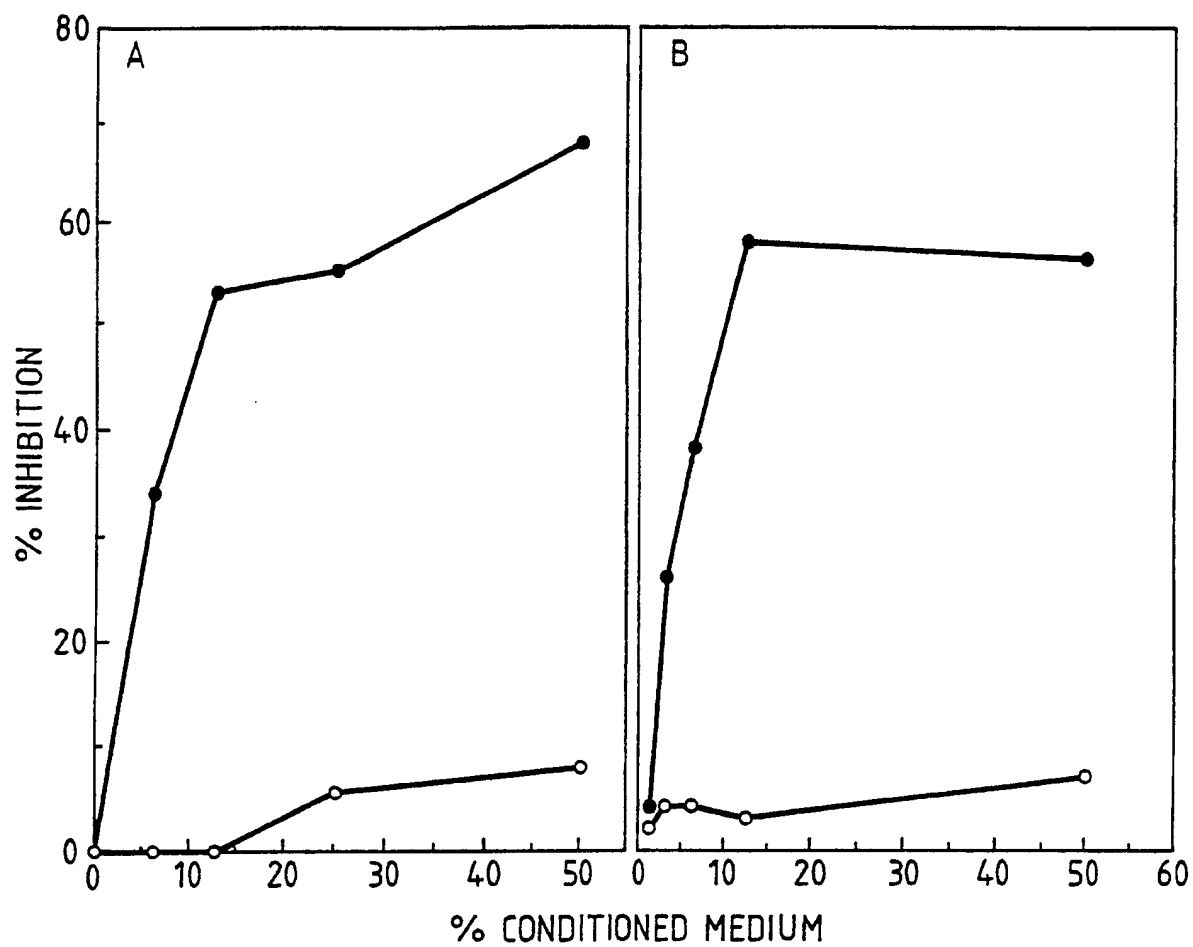
Fig. 2.

4|13

Fig.3.

Fig.4.

Fig. 5.
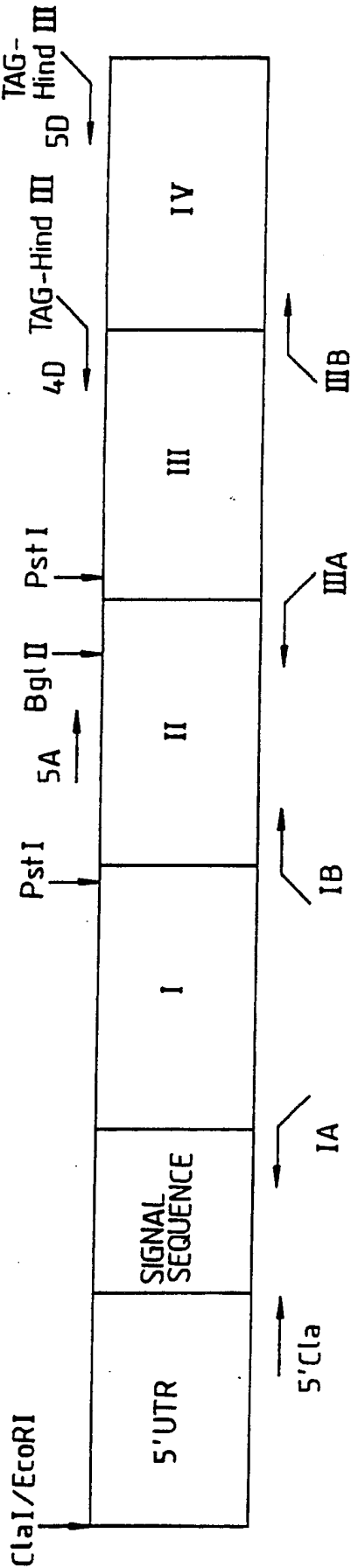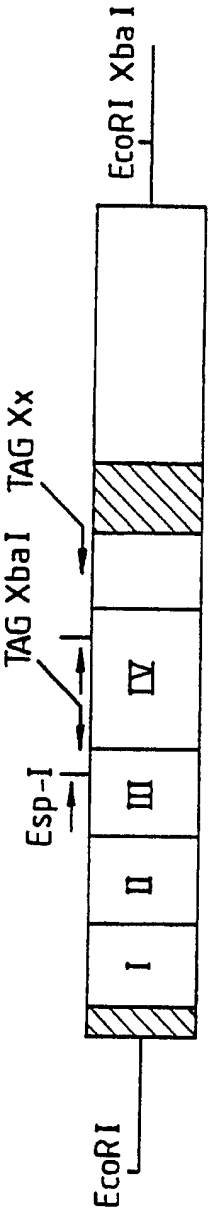
Fig.13.

Fig.6.

First Subdomain
TNFR-55,
TNFR-75,
NGFR,
CD40,
OX40,

Second Subdomain
TNFR-55,
TNFR-75,
NGFR,
CD40,
OX40,

Third Subdomain
TNFR-55,
TNFR-75,
NGFR,
CD40,

Fourth Subdomain
TNFR-55,
TNFR-75,
NGFR,
CD40,
OX40,

Fig. 7.

DNA sequence   608 b.p.   TGTCTGGCATGG ... CCCAGATTTAG   linear

```
9  / 1                                                                          39 / 11
ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CCG CTG CTC CTG GAG CTG TTG GTG
met gly leu ser thr val pro asp leu leu pro leu val leu glu leu leu val
69 / 21                                                                         99 / 31
GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG AAG AGA
gly ile tyr pro ser gly val ile gly leu val pro his leu gly asp arg lys arg
129 / 41                                                                        159 / 51
GAT AGT GTG TGT CCC CAA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC
asp ser val cys pro gln gly lys tyr ile his pro gln asn asn ser ile cys cys thr
189 / 61                                                                        219 / 71
AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT GAC TGC GGG CAG GGG CCG GAT ACG GAC
lys cys his lys gly thr tyr leu tyr asn asp cys asp gly pro gly gln thr asp
249 / 81                                                                        279 / 91
TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC
cys arg glu cys glu ser gly ser phe thr ala ser glu asn his leu arg his cys leu
309 / 101                                                                       339 / 111
AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC
ser cys ser lys cys arg lys glu met gly val glu ile ser ser cys thr val asp
369 / 121                                                                       399 / 131
CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT
arg asp thr val cys gly cys arg lys asn gln tyr arg his tyr trp ser glu asn leu
429 / 141                                                                       459 / 151
TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG
phe gln cys phe asn cys ser leu cys leu asn gly thr val his leu ser cys gln glu
489 / 161                                                                       519 / 171
AAA CAG AAC ACC GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG GTG TGT GTC
lys gln asn thr val cys thr cys his ala gly phe phe leu arg glu asn glu cys val
549 / 181                                                                       579 / 191
TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG TGC CTA CCC CAG ATT TAG
ser cys ser asn cys lys lys ser leu glu cys thr cys leu pro gln ile AMB
```

Fig. 8.

```
DNA sequence    482 b.p.    TGTCTGGCATGG ... CCCCAGATTTAG    linear

9 / 1                                          39 / 11
ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG    CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG
met gly leu ser thr val pro asp leu leu    leu pro leu val leu leu glu leu leu val
69 / 21

GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG    GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA
gly ile tyr pro ser gly val ile gly leu    val pro his leu gly asp arg glu lys arg
129 / 41                                        99 / 31

GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA    GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC
glu cys glu ser gly ser phe thr ala ser    glu asn his leu arg his cys leu ser cys
189 / 61                                        159 / 51

TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG    GAG ATC TCT TCT TGC ACA GTG GAC CGG GAC
ser lys cys arg lys glu met gly gln val    glu ile ser ser cys thr val asp arg asp
249 / 81                                        219 / 71

ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC    CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG
thr val cys gly cys arg lys asn gln tyr    arg his tyr trp ser glu asn leu phe gln
309 / 101                                       279 / 91

TGC TTC AAT TGC AGC CTC TGC CTC AAT GGG    ACC GTG CAC CTC TCC TGC CAG AAA CAG
cys phe asn cys ser leu cys leu asn gly    thr val his leu ser cys gln lys gln
369 / 121                                       339 / 111

AAC ACC GTG TGC ACC TGC CAT GCA GGT TTC    TTT CTA AGA GAA AAC GAG CTG GAG TGT GTC
asn thr val cys thr cys his ala gly phe    phe leu arg glu asn glu leu glu cys val
429 / 141                                       399 / 131

AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG    AAG TTG TGC CTA CCC CAG ATT TAG
ser asn cys lys lys ser leu glu cys thr    lys leu cys leu pro gln ile AMB
                                                459 / 151
```

10/13

Fig. 9.

DNA sequence   470 b.p.   TGTCTGGCATGG .... CCCCAGATTTAG   linear

```
 9 /   1                            39 /  11
ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG
met gly leu ser thr val pro asp leu leu leu pro leu val leu leu glu leu leu val
69 /  21                            99 /  31
GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG CTC CCT CAC GTC CTA GGG GAC AGG GAG AAG AGA
gly ile tyr pro ser gly val ile gly leu leu pro his leu val pro gly asp arg glu lys arg
129 /  41                           159 /  51
GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC
asp ser val cys pro gln gly lys tyr ile his pro gln asn asn ser ile cys cys thr
189 /  61                           219 /  71
AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC
lys cys his lys gly thr tyr leu tyr asn asp cys pro gly pro gly gln asp thr asp
249 /  81                           279 /  91
TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TGC TTC AAT TGC
cys arg lys asn gln tyr arg his tyr trp ser glu asn leu phe gln cys cys phe asn cys
309 / 101                           339 / 111
AGC CTC TGC AAT GGG ACC GTG CAC CTC TGC CAG GAG AAA CAG AAC ACC GTG TGC
ser leu cys asn gly thr val his leu cys gln glu lys gln asn thr val cys
369 / 121                           399 / 131
ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC TCC TGT AGT AAC TGT AAG
thr cys his ala gly phe phe leu arg glu asn glu cys val ser cys ser asn cys lys
429 / 141                           459 / 151
AAA AGC CTG GAG TGC ACG AAG TTG TGC CTA CCC CAG ATT TAG
lys ser leu glu cys thr lys leu cys leu pro gln ile AMB
```

Fig.10.

DNA sequence 485 b.p.   TGTCTGGCATGG ... CCCCAGATTTAG   linear

```
9  /  1
ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG CTC CTG GAG CTG TTG GTG
met gly leu ser thr val pro asp leu leu leu pro leu leu leu glu leu leu val
69 /  21
GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA
gly ile tyr pro ser gly val ile gly leu val pro his leu gly asp arg glu lys arg
129 / 41
GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC
asp ser val cys pro gln gly lys tyr ile his pro gln asn asn ser ile cys cys thr
189 / 61
AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG CAG GAT ACG GAC
lys cys his lys gly thr tyr leu tyr asn asp cys pro gly pro gln thr asp asp
249 / 81
TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC
cys arg glu cys glu ser gly ser phe thr ala ser glu asn his leu arg his cys leu
309 / 101
AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GTG ATC TCT TGC ACA GTG GAC
ser cys ser lys cys arg lys glu met gly gln val glu ile ser cys thr val asp
369 / 121
CGG GAC ACC GTG TGT ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC TCC
arg asp thr val cys thr cys his ala gly phe phe leu arg glu asn glu cys val ser
429 / 141
TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TGC CTA CCC CAG ATT TAG
cys ser asn cys lys lys ser leu glu cys thr lys leu cys leu pro gln ile AMB
```
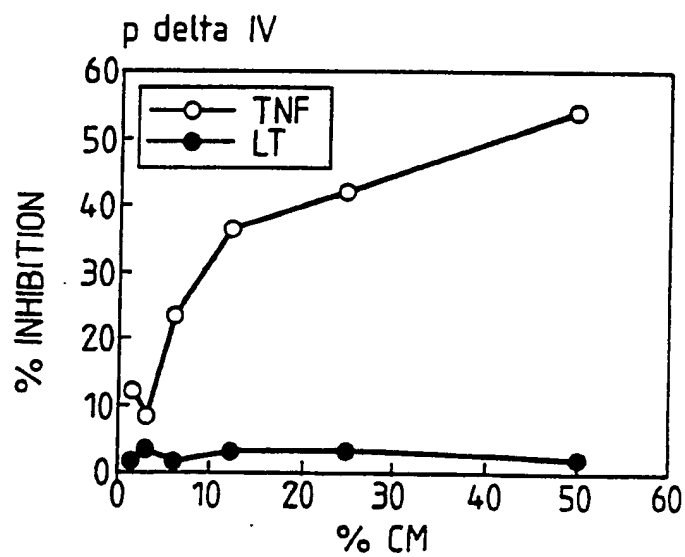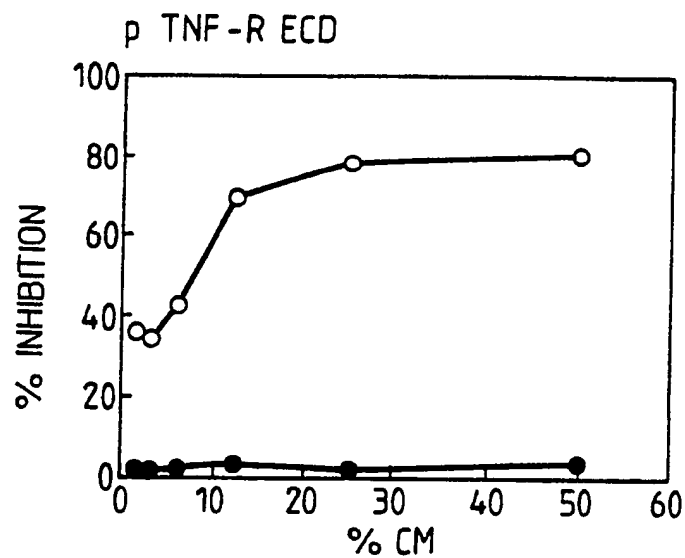
12/13

Fig.11.

DNA sequence   512 b.p.   TGTCTGGCATGG ... GTGTGCACCTGA   linear

```
9    /    1                               39    /    11
ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CTC CTG GAG CTG TTG GTG
met gly leu ser thr val pro asp leu leu leu val leu leu glu leu leu val

69   /   21                               99    /    31
GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA
gly ile tyr pro ser gly val ile gly leu val pro his leu gly asp arg glu lys arg

129  /   41                              159   /    51
GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC
asp ser val cys pro gln gly lys tyr ile his pro gln asn asn ser ile cys cys thr

189  /   61                              219   /    71
AAG TGC CAC AAA GGA ACC TAC TTG TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC
lys cys his lys gly thr tyr leu tyr leu tyr asn asp cys pro gly pro gly gln asp thr asp

249  /   81                              279   /    91
TGC AGG GAG TGT GAG AGC GGC TCC ACC TTC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC
cys arg glu cys glu ser gly ser thr phe ala ser glu asn his leu arg his cys leu

309  /  101                              339   /   111
AGC TGC TCC AAA GAA ATG GGT CAG GTG GTG GAG ATC TCT TGC ACA GTG GAC
ser cys ser lys glu met gly gln val glu ile ser cys thr val asp

369  /  121                              399   /   131
CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG CAC CGG CAT TAT TGG AGT GAA AAC CTT
arg asp thr val cys gly cys arg lys asn gln tyr arg his tyr trp ser glu asn leu

429  /  141                              459   /   151
TTC CAG TGC TTC AAT TGC AGC CTC CTC TGC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG
phe gln cys phe asn cys ser leu leu cys asn gly thr val his leu ser cys gln glu

489  /  161
AAA CAG AAC ACC GTG TGC ACC TGA
lys gln asn thr val cys thr OPA
```

SUBSTITUTE SHEET

Fig.12.

p TNF-R ECD

p delta IV

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/12;   C07K13/00;   A61K37/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[°]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP,A,0 308 378 (YEDA RESEARCH AND DEVELOPMENT COMPANY, LIMITED) 22 March 1989 see the whole document ---	1-14
X	CELL. vol. 61, 20 April 1990, CAMBRIDGE, NA US pages 351 - 359; Shall, T.J. et al.: 'Molecular cloning and expression of the human 55Kd tumor necrosis factor receptor.' see the whole document ---	1-14
X	CELL. vol. 61, 20 April 1990, CAMBRIDGE, NA US pages 361 - 370; Loetscher, H. et al.: 'Molecular cloning and expression of a receptor for human tumor necrosis factor.' see the whole document --- -/--	1-14

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23 JANUARY 1992	0 6. 02. 92

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	NAUCHE S.A.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 87, 1 October 1990, WASHINGTON US pages 7380 - 7384; Gray, Patrick W.; Barrett, Kathy; Chantry, David; Turner, Martin; Feldmann, Marc: 'Cloning of human tumor necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein' see the whole document ---	1-14
P,X	EP,A,0 393 438 (BOEHRINGER INGELHEIM INTERNATIONAL) 24 October 1990 see the whole document ---	1-14

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 23/01/92

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0308378	22-03-89	AU-A-	2206888	16-03-89
		JP-A-	2000200	05-01-90
EP-A-0393438	24-10-90	DE-A-	3913101	31-10-90
		DE-A-	3920282	03-01-91
		JP-A-	3164179	16-07-91